Processing of chimeric mammalian cytochrome b_5 precursors in *Escherichia coli*: reaction specificity of signal peptidase and identification of an aminopeptidase in post-translocational processing

Victoria HARDING, Amna KARIM,* Naheed KADERBHAI, Adam JONES, Alun EVANS and Mustak A. KADERBHAI†
Department of Biochemistry, The University of Wales, Penglais, Aberystwyth, Dyfed, SY23 3DD, U.K.

A chimeric precursor interlinked by an arginine residue between the full-length signal sequence of alkaline phosphatase and the eukaryotic cytoplasmic cytochrome b_5 was constructed. Expression of the chimeric precursor protein in *Escherichia coli* resulted in efficient export of spectrally authentic cytochrome b_5 into the periplasm [Karim, Harding, Evans, Kaderbhai and Kaderbhai (1993) Bio/Technology **11**, 612–618]. On sequencing, the apparent absence of arginine at the N-terminus of the secreted cytochrome b_5 implied that the chimera was either miscleaved by signal peptidase or further processed following signal excision by an uncharacterized peptidase. The influence of the N-terminal region of cytochrome b_5 on the unusual processing of the chimeric precursor was investigated by engineering a number of variant forms in which the region between Arg^{+1} and the mature portion of cytochrome b_5 was extended and varied. Observations of the *in vivo* processed patterns of these variant cytochrome b_5 forms exported into the periplasm revealed that the absence of arginine was due to neither miscleavage of the translocated precursor by the signal peptidase nor the nature of the early region of cytochrome b_5. In fact, the selective excision of the arginine residue occurred subsequent to signal sequence deletion by an aminopeptidase which was sensitive to the metal chelator *o*-phenanthroline. We show that this aminopeptidase also participates in the trimming of the N-terminal arginine residue of the bacterial alkaline phosphatase to generate the three isoenzymes in the periplasm.

INTRODUCTION

The production of recombinant proteins by secretion or export in bacteria not only provides a means of producing significant quantities of functional biomolecules, but also represents a way of investigating both the mechanism of protein translocation across the cytoplasmic membrane and the associated post-translocational modifications. The prokaryotic post-translational post-translocational machinery is generally thought to be less complex than that involved in the secretory pathway in eukaryotes. Nevertheless, most proteins translocated across the bacterial cytoplasmic membrane require cleavage of the signal sequence (Wickner et al., 1991) and often undergo modifications involving further proteolysis (Dev and Ray, 1990), disulphide bond formation (Derman and Beckwith, 1991) and linkage with prosthetic groups (Karim et al., 1993). Except for signal peptidases I and II (Muller, 1992), many of the periplasmic catalysts involved in the modifications of exported proteins are poorly characterized in *Escherichia coli*.

Recently we have reported on the efficient expression and secretion of an artificial chimeric precursor of cytochrome b_5 in *E. coli* (Karim et al., 1993). This chimeric precursor, SS-R-b_5, represents fusion between the alkaline phosphatase (AP) signal sequence (SS) and the soluble core domain of rat liver cytochrome b_5 (b_5), interlinked by Arg^{+1} of AP. The chimera was placed under the transcriptional control of the native *pho* promoter. We chose the full-length 21-residue SS for tailoring the exportable b_5 because it shows the classic characteristics of known signal sequences. In common with most signals, SS has a basic N-terminus, a central apolar region and a basic C-terminus. The placement of Arg^{+1} of AP immediately prior to the Met^{+1} of b_5 was prompted by the findings of Li et al. (1988) that the nature and the distribution of the charged residues at the N-terminus of AP critically influenced the export of SS-AP. The inclusion of Arg^{+1} in the chimeric precursor in turn displaced the Glu^{+3} of b_5 to the corresponding position of the same residue in SS-AP. The prokaryotic SS selectively targeted SS-R-b_5 to the periplasm, where it was processed to yield significant amounts (6 mg/litre of culture) of correctly matured, haem-assembled b_5. Structurally and spectrally, the exported haemoprotein was indistinguishable from the authentic trypsin-cleaved b_5 of the liver endoplasmic reticulum. However, the exported precursor was predominantly processed immediately before the Met^{+1} of b_5, a surprising finding because the expected cleavage point, preceding the arginine, was surpassed by one residue. The $-3,-1$ scission rule of von Heijne (1990) specifies that only small, neutral amino acids are accommodated at $-3,-1$ positions relative to the cleavage site of the bacterial signal peptidase. The apparent 'miscleavage' may have occurred due to (i) the nature of the early N-terminal sequence of the unnaturally secreted b_5, or (ii) selective deletion of the Arg^{+1} residue (also from the *phoA* product), subsequent to signal peptide cleavage, presumably by a peptidase not related to signal peptidase.

It was, therefore, of interest to investigate whether the reaction specificity of the signal peptidase was affected by the early mature portion of the unnaturally secreted b_5. By engineering a

Abbreviations used: AP, alkaline phosphatase; b_5, soluble cytochrome b_5 core; SS, signal sequence; SS-R-b_5, precursor fusion between SS and cytochrome b_5 linked by an arginine residue; SS-R-X_n-b_5, variants of SS-R-b_5 with peptide link X_n, where X is an amino acid residue in the sequence *n*.

* Present address: Department of Biochemistry, Faculty of Medicine, King Abdul Aziz University, Jeddah, Saudi Arabia.
† To whom correspondence should be addressed.

number of chimeric b_5 precursors (SS-R-X_n-b_5) carrying defined peptide extensions (X_n, where X is the amino acid residue in the sequence n) positioned beyond the cleavage site and immediately prior to b_5, we show that the reaction specificity of *E. coli* signal peptidase in the processing of SS-R-X_n-b_5 is in accordance with the established $-3,-1$ rule. The apparent miscleavage, beyond the terminal signal residue, was brought about by selective post-translocational processing of the Arg^{+1} by an aminopeptidase activity that is sensitive to metal chelation. We show that this putative metallopeptidase plays a role in formation of the three isoenzymes of alkaline phosphatase.

MATERIALS AND METHODS

Plasmids, bacteria and culture conditions

The plasmid pAF-cyt (Karim et al., 1993) was the progenitor for the constructs coding for the secreted forms of b_5 mutants described here. The molecular biological procedures were essentially as described by Maniatis et al. (1989). Site-directed mutagenesis involved replacing the restriction enzyme-deleted DNA duplexes from plasmid DNA with synthetic oligonucleotides. Mutations were confirmed by DNA sequencing (Sanger et al., 1977). All vectors were propagated in *E. coli* strain TB-1. The induction of the *pho* promoter was triggered by growth of bacteria in a phosphate-limited Mops medium with 75 μg/ml ampicillin at 35 °C for 6 h; a 2% Luria broth culture grown to saturation served as a starter. Periplasmic content was fractionated (Karim et al., 1993) from *E. coli* cultivated in Mops medium for 6 h.

Isolation, sequencing and analysis of b_5 and X_n-b_5

Homogeneous, holo forms of b_5 and X_n-b_5 (50–100 μg) were isolated by preparative electrophoresis of periplasmic proteins in non-denaturing 14% polyacrylamide gels, employing the conditions described by Gallagher and Smith (1991); the colour and the significantly faster mobility of the recombinant proteins over the remaining periplasmic proteins facilitated their isolation (see Figure 4). The purity of the haemoprotein species, assessed by measuring their specific content, exceeded 95%. N-terminal sequence analyses were performed on an Applied Biosystem 473A sequencer (Alta Bioscience, Birmingham University, U.K.). Electrophoretic analysis of the periplasmic proteins were conducted in 14% polyacrylamide gels, with approx. 150 μg of protein loaded in each track. Unless stated otherwise, the proteins were detected by staining with Coomassie Blue.

Assays

All of the X_n-b_5 forms described in this study were spectrally identical to the native b_5 (Gallagher et al., 1992), and were quantified (oxidized state) from the Soret absorption peak at 413 nm using $\epsilon = 115\ mM^{-1} \cdot cm^{-1}$ (Hultquist, 1978). Where bovine haem was used as a supplement to convert apo- to holo-haemoproteins, it was added as a stock 1 mM solution [0.1 M Tris/HCl, pH 8.2, 80% (v/v) ethylene glycol] in equal amounts to the contents of both the test and reference cuvettes so as to give final concentrations ranging from 2.5 to 20 μM. Protein was estimated by the procedure of Bradford (1976) using BSA as the standard.

RESULTS AND DISCUSSION

Processing of SS-R-I-X-Q-b_5 precursors

The reaction of signal peptidases specifies that only small, neutral amino acids are accommodated at $-3,-1$ positions relative to the cleavage site. In addition, a β-turn located 4–6 residues downstream is important for correct alignment of the $-3,-1$ residues at the cleavage site. While the cleavage of SS from native SS-AP has been predicted to conform to the $-3,-1$ rule (Chang et al., 1986), SS-R-b_5 processing (see Figure 3) would appear to breach the rule in that both the $-3,-1$ residues carry larger, charged side groups. Although there are no strict limitations on the choice of the $+1$ residue for cleavage by signal peptidase I, the apparent preference for Met^{+1} (b_5) in the processing of SS-R-b_5 demonstrated yet another dichotomy, in that this residue has, so far, not been found in prokaryotic precursors (Barkocy-Gallagher and Bassford, 1992); indeed, all 20 amino acids are found in this position in eukaryotic pre-proteins. Was the apparently unusual processing of SS-R-b_5 a consequence of the immediate N-terminal sequence of b_5? To investigate this, three SS-R-X_n-b_5 precursors were constructed in which the tripeptide sequence I-X-Q was engineered between Arg^{+1} of AP and Met^{+1} of b_5 (Figure 1). These SS-R-I-X-Q-b_5 precursors were adequately expressed in *E. coli* and exported to the periplasm (Figure 2). Measurement of the periplasmic content of the haemoproteins derived from SS-R-I-X-Q-b_5 showed a biphasic trend, in which an early phase of accumulation until the mid-exponential period was followed by a gradual decline extending up to 14 h of growth. The alteration of a single amino acid residue within the tripeptide sequence of SS-R-I-X-Q-b_5 had a small but significant effect on the eventual yield of the exported protein. At peak build-up, the SS-R-I-X-Q-b_5 haemoproteins have a 2-fold lower export than the b_5 derived from SS-R-b_5, suggesting that the rates of translocation and/or processing of SS-R-I-X-Q-b_5 may have been impeded by the tripeptide extensions. Over the first 6–8 h of culture growth, the changes in total periplasmic protein content of the three cell lines were very similar. At the stationary phase, the only difference in periplasmic protein concentration was

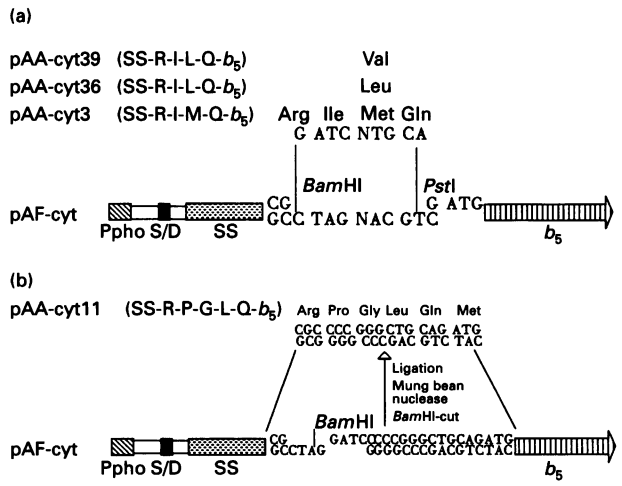

Figure 1 Construction of plasmids

(**a**) Plasmids expressing SS-R-I-X-Q-b_5 was constructed by ligating the synthetic nonanucleotide, carrying a random base at the fifth position, into *Bam*HI–*Hin*dIII-cleaved pAF-cyt. (**b**) A plasmid expressing SS-R-P-G-L-Q-b_5 was constructed by flush-ending the *BAm*HI-cleaved pAF-cyt and recircularization. Abbreviations: Ppho, *pho* promoter; S/D, Shine/Dalgarno sequence.

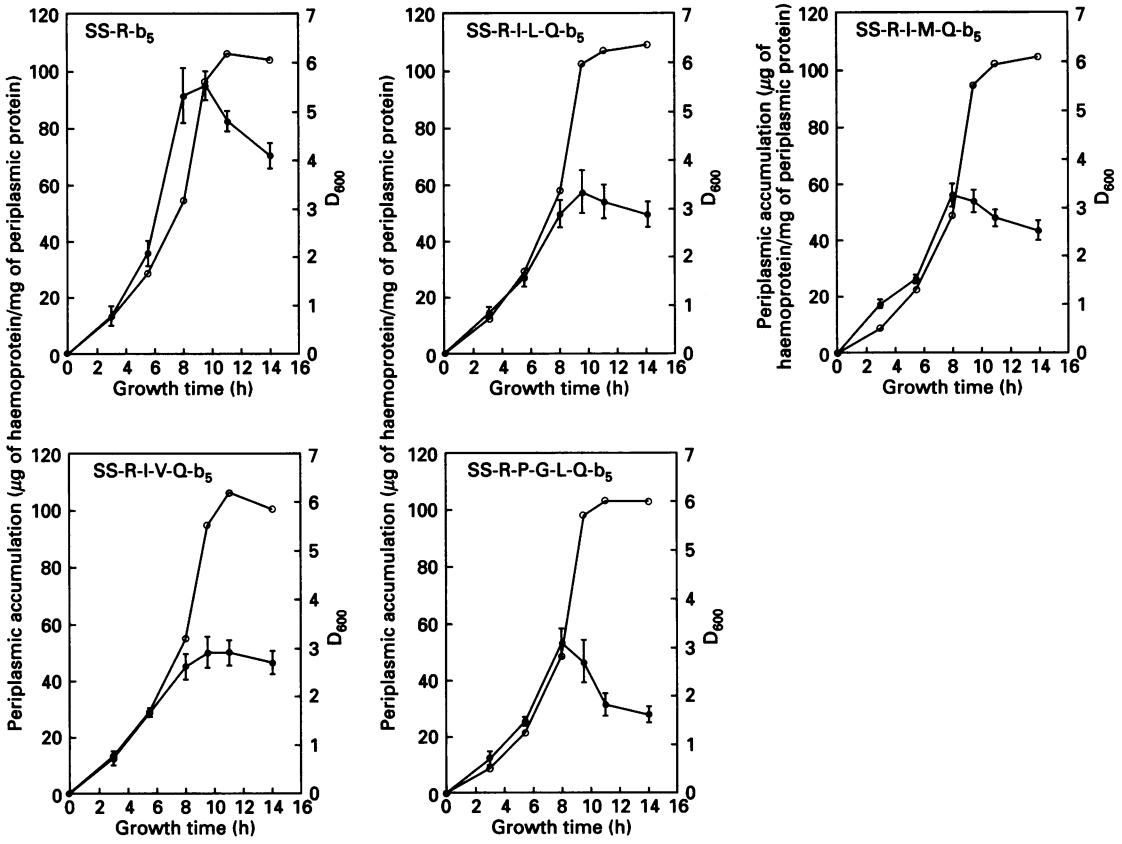

Figure 2 Time courses of periplasmic accumulation of b_5 and X_n-b_5

E. coli cultures (20 ml) cultivated in Mops for 6 h were harvested and the periplasmic fractions prepared as described by Karim et al. (1993). The data are derived from two separate experiments. ●, Spectrally estimated holo-form of the haemoproteins expressed per mg of periplasmic protein (means ± S.E.M.); ○, culture density, measured as attenuance (*D*), at 600 nm.

	−5	−4	−3	−2	−1	⇓1AP	2AP	3AP	4AP	5AP	6AP	7AP	8AP	9AP
SS-AP	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	Thr−	Pro−	Glu−	Met−	Pro−	Val−	Leu−	Glu−
	−5	−4	−3	−2	−1	1AP								
SS-R-X_7 (pAF-cyt)	Pro	Val	Thr	Lys	Ala	Arg	Ile	Pro	Arg	Ala	Ala	Asp	Gly	End
	−5	−4	−3	−2	−1	1AP	⇓1b5	2b5	3b5	4b5	5b5	6b5	6b5	7b5
SS-R-b_5 (pAA-cyt)	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	Met−	Ala−	Glu−	Gln−	Ser−	Asp−	Lys	Asp
	−5	−4	−3	−2	−1	1AP	⇓			1b5	2b5	3b5	4b5	5b5
SS-R-I-M-Q-b_5 (pAA-cyt3)	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	*Ile−*	*Met−*	*Gln−*	Met−	Ala−	Glu−	Gln−	Ser−
	−5	−4	−3	−2	−1	1AP	⇓			1b5	2b5	3b5	4b5	5b5
SS-R-I-L-Q-b_5 (pAA-cyt36)	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	*Ile−*	*Leu−*	*Gln−*	Met−	Ala−	Glu−	Gln−	Ser−
	−5	−4	−3	−2	−1	1AP	⇓			1b5	2b5	3b5	4b5	5b5
SS-R-I-V-Q-b_5 (pAA-cyt39)	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	*Ile−*	*Val−*	*Gln−*	Met−	Ala−	Glu−	Gln−	Ser−
	−5	−4	−3	−2	−1	⇓	1AP	⇓			1b5	2b5	⇓	3b5	4b5
SS-R-P-G-L-Q-b_5 (pAA-cyt11)	Pro−	Val−	Thr−	Lys−	Ala−	Arg−	*Pro−*	*Gly−*	*Leu−*	*Gln−*	Met−	Ala−	Glu−	Gln−	

Figure 3 N-Terminal sequences of SS-AP, SS-R-b_5 and SS-R-X_n-b_5

The (common) wild-type sequences are underlined. The terminal residue of SS is Ala^{-1} and all constructs carried Arg^{+1} of AP. The engineered residues are in italics. The sequences of the wild-type SS-AP and the SS-R-X_7 (encoded by the clone pAF-cyt and used as the control) are included for comparison. Arrows designate the eventual processing sites of the exported precursors deduced from the N-terminal sequences of the exported b_5 species.

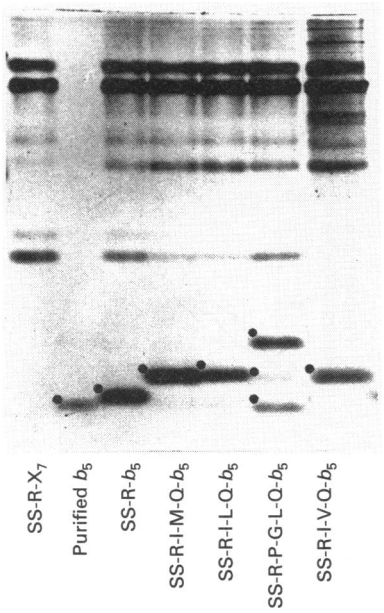

Figure 4 Non-denaturing gel electrophoresis of periplasmic fractions of *E. coli* expressing SS-R-b_5 and SS-R-X_n-b_5

SS-R-X_7 (pAF-cyt-encoded) served as the control. The dots mark the positions of the respective forms of the secreted haemoproteins, identified by their intense brown colour prior to staining with Coomassie Blue. Note that the purified non-secretory form of b_5 (5 μg), isolated from the cytoplasm of *E. coli* pλ-1cyt (Gallagher et al., 1992), lacks the N-terminal methionine.

between the SS-R-I-X-Q-b_5-exported haemoproteins and those derived from SS-R-b_5. Thus the affected rates of translocation and/or processing may have been influenced at the stationary phase. Alternative explanations for the different pattern of haemoprotein accumulation at the stationary phase may be attributed to a lesser production/stability of b_5 protein in SS-R-I-X-Q-b_5-expressing cells than that in cells expressing SS-R-b_5. Therefore, selective action of proteinase(s) could determine the levels of variant forms of the haemoproteins at different growth phases.

The N-terminal sequences of the exported haemoproteins registered the presence of the entire tripeptide sequence in all three of the processed derivatives (Figure 3). The periplasmically derived I-X-Q-b_5, shown resolved in the native gel in Figure 4, consistently migrated at a slower rate than b_5, and the banding pattern remained unaltered throughout the growth phase (results not shown). The finding that all three variants of SS-R-I-X-Q-b_5 precursors were eventually processed at the R-I peptide bond indicated that the nature of the immediate N-terminal sequence of the haemoprotein was not a contributory factor in the apparently unusual processing of the unnaturally secreted b_5. However, the retention of the additional three amino acid residues in all of the exported I-X-Q-b_5 complicated the proposition of the involvement of an aminopeptidase after signal excision, unless such a putative peptidase was specific for arginine or basic residue at the N-terminus.

An SS-R-P-G-L-Q-b_5 precursor is processed according to the $-3,-1$ rule

With the exception of some retroviral proteinases, it is extremely uncommon for a proteinase or a peptidase to cleave an X-P bond (Oroszlan and Luftig, 1990). Proline is an imino acid whose R group is covalently bonded to the α-amino group, forming a cyclic structure that is incorporated into the peptide chain. The peptide imine is not readily available to accept a proton in general acid–base catalysis, a common mechanism for proteinase cleavage. Hence the precursor SS-R-P-G-L-Q-b_5 was engineered with placement of a proline residue following the Arg^{+1}. This strategy would abort peptide cleavage at the R-P bond and in turn reveal the true nature of processing by the signal peptidase. Bacterial expression of the SS-R-P-G-L-Q-b_5 precursor resulted in the export of haemoprotein at a level comparable with that of I-X-Q-b_5 at around the mid-exponential growth phase (Figure 2). The time-course profile of the exported haemoprotein from SS-R-P-G-L-Q-b_5 displayed a similar trend to that of SS-I-X-Q-b_5, but in the second phase (past mid-stationary period) it displayed poorer stability. These findings, in general, imply that the composition of the X_n sequence in SS-R-X_n-b_5 constructs has varying effects at more than one stage of the export and assembly processes, and influences the rate of proteolysis of the secreted proteins.

Electrophoretic analysis revealed that the exported haemoproteins derived from SS-R-P-G-L-Q-b_5 were processed in three different ways (Figures 3 and 4); qualitatively, the triplet banding pattern remained unaltered during the entire growth phase (results not presented). More importantly, the major processed form of b_5 (Figure 4, upper band), accounting for about 65% of the total exported haemoprotein, was cleaved prior to the arginine residue. The middle band, constituting about 5% of the exported protein, was derived from cleavage of the R-P bond. Thus the reaction specificity of the signal peptidase became apparent when Arg^{+1} was placed adjacent to proline, as the putative amino peptidase would not effectively cleave the Arg-Pro imido peptide bond.

The derivation of the second major species (Figure 4, lower band) of the processed b_5 (constituting about 30% of the exported protein) from the cleavage of SS-R-P-G-L-Q-b_5 at the new position between alanine and glutamine, six residues upstream from the terminal Ala^{-1} of SS (see Figure 3), could be explained in the light of the known reaction specificity of bacterial signal peptidase. The proposition by Perlman and Halvorson (1983) that proper juxtaposition of the cleavage site with signal peptidase depends strongly on its location relative to the origin of the turn has been convincingly demonstrated for the eukaryotic signal peptidase (Nothwehr and Gordon, 1989). In prokaryotic signal peptides the cleavage site is typically located 5–6 residues from the end of the core. Thus for SS-R-P-G-L-Q-b_5 the second cleavage site could potentially arise from recognition of glutamine and alanine as the $-3,-1$ residues, ideally placed five residues from the proline introduced in the engineered tetrapeptide sequence linking with b_5. Consistent with the previous findings of Fikes et al. (1990) on the effect of substituting amino acid residues at the -3 position on the processing of premaltose-binding protein, the location of the bulky glutamine residue at the -3 position in the new processing site in SS-R-P-G-L-Q-b_5 could explain the significantly reduced efficiency of scission by signal peptidase. However, this interpretation is marred by the observation that the derivation of the second processed species is completely abolished by inhibitors of proteinases which do not affect signal peptidase activity (see below and Figure 6a).

Arginine processing of R-b_5, R-X_n-b_5 and R-AP

We investigated the potential involvement of a peptidase in the post-signal peptidase processing of the N-terminal arginine of the bacterially secreted R-b_5 by treating cultures expressing SS-

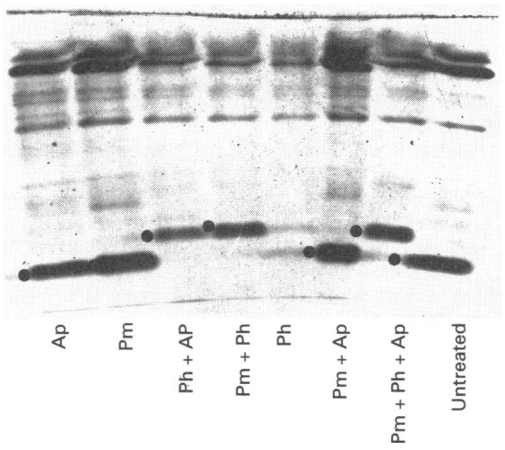

Figure 5 Effect of proteinase inhibitors on the *in vivo* processing of the N-terminal arginine of the secreted R-b_5

Individual cultures of *E. coli* (20 ml) expressing SS-R-b_5 were untreated (control) or treated with three doses of 75 units/ml aprotinin (Ap) and/or 0.25 mM phenylmethanesulphonyl fluoride (Pm) and/or 0.25 mM *o*-phenanthroline (Ph) at 3, 4 and 5 h of the growth phase. Periplasmic proteins (150 μg/track) were analysed for the secreted haemoproteins in the standard non-denaturing polyacrylamide gel. The upper and the lower dots mark the positions of R-b_5 and b_5 respectively.

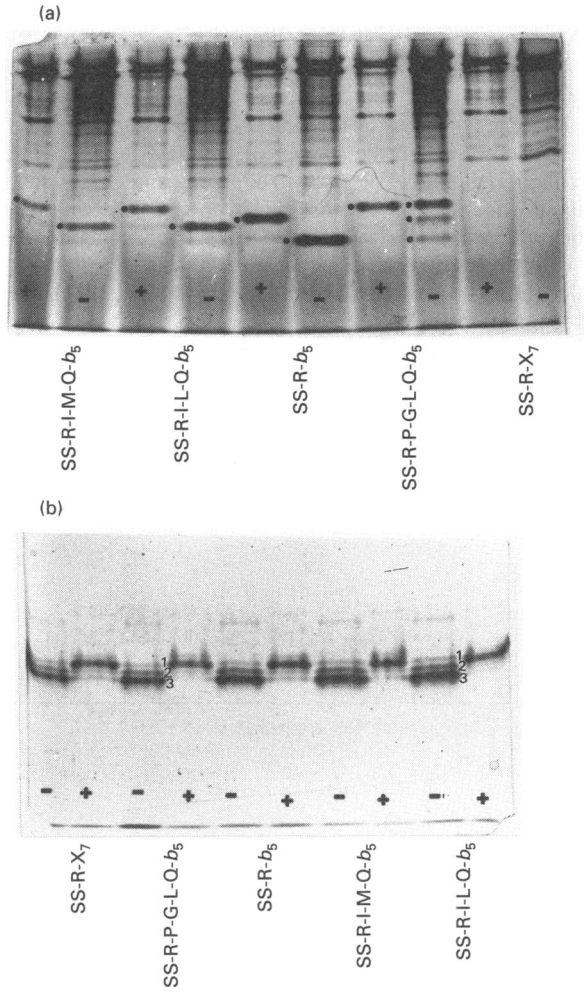

Figure 6 *In vivo* inhibition of processing of the N-terminal arginine of secreted R-b_5, R-X_n-b_5 and R-AP

Individual cultures (20 ml) of *E. coli*, synthesizing the above recombinant (haemo)proteins, were either untreated (−) or treated (+) with a combined dose of 0.25 mM *o*-phenanthroline, 0.25 mM phenylmethanesulphonyl fluoride and 75 units/ml aprotinin at 3, 4 and 5 h of the growth phase. The periplasmic proteins were analysed by separation in non-denaturing polyacrylamide gels. (**a**) Proteins seen by staining with Coomassie Blue after separation in a 14% gel. The upper and lower dots mark the respective positions of the arginine-unprocessed and arginine-processed haemoproteins. (**b**) Bands detected by assaying AP activity after incubating the gel (7.5% polyacrylamide) with 0.5 mg/ml α-naphthyl pyrophosphate and 0.5 mg/ml 4-chloro-*o*-toluidinediazonium in 30 mM Tris/HCl (pH 9). Bands 1, 2 and 3 are the respective dimers R-AP:R-AP, R-AP:AP and AP:AP.

R-b_5 with inhibitors of proteinases (Figure 5). The serine proteinase inhibitors aprotinin and phenylmethanesulphonyl fluoride, applied either singly or in combination, did not affect the electrophoretic mobility of the exported b_5. However, the metal chelator *o*-phenanthroline caused a dramatic inhibition of the periplasmic accumulation of b_5 and generated an additional, slower moving, coloured haemoprotein band, subsequently identified as R-b_5 by N-terminal sequencing of the isolated protein. The treatment of bacteria with *o*-phenanthroline in combination with the serine proteinase inhibitors substantially improved periplasmic recovery of R-b_5. The same treatment of bacteria expressing the mutant SS-R-X_n-b_5 chimeras resulted, in each case, in the disappearance of the exported X_n-b_5 species (uninhibited state) and concomitant appearance of a prominent slower migrating band, subsequently identified as R-X_n-b_5 forms (Figure 6a).

Clearly, these findings confirm that the reaction specificity of the bacterial signal peptidase in the cleavage of SS-R-X_n-b_5 conforms with the $-3,-1$ rule and is unaffected by the upstream sequences in the mature portion of b_5. The data further demonstrate that the subsequent removal of the N-terminal arginine is brought about by an uncharacterized metallopeptidase which may be specific for arginine. The enhanced stability of R-b_5 (and R-X_n-b_5) in bacteria treated with *o*-phenanthroline and serine proteinase inhibitors suggests that the exported R-b_5, unless converted to b_5 by the metallopeptidase, is susceptible to degradation by serine proteinase(s). Although *o*-phenanthroline exerted significant inhibition on the overall productivity of many of the periplasmic proteins (Figure 6a), this is not likely to arise from inhibition of signal peptidase, which has been reported to be insensitive to a wide variety (over 30) of commercially available inhibitors, including *o*-phenanthroline, phenylmethanesulphonyl fluoride and aprotinin (Black et al., 1992).

It is pertinent to ask whether the post-translocational processing of arginine of SS-R-X_n-b_5 is of any physiological significance. Indeed, *E. coli* AP occurs in three dimeric forms, designated isoenzymes 1, 2 and 3. Although at first dismissed as impurities in the preparations (Bridgen and Secher, 1973), later studies (Schlesinger et al., 1975) showed that these forms differ by the presence of an arginine residue at the N-terminus on the subunits of isoenzyme 1 (R-AP:R-AP) and the absence of the same in isoenzyme 3 (AP:AP). Isoenzyme 2 is a heterodimer in which one subunit has the N-terminal arginine (R-AP:AP). Based on their charge differences resulting from the number of N-terminal arginine residues, the three isoenzymes of AP can be electrophoretically resolved under non-denaturing conditions and identified by AP activity staining (Figure 6b). As observed with the processing of the SS-R-b_5 and SS-R-X_n-b_5, *in vivo* treatment of *E. coli* cultures with *o*-phenanthroline, together with phenylmethanesulphonyl fluoride and aprotinin, caused an

almost complete shift in the banding of the dominant periplasmic AP:AP form (found in the uninhibited state) to R-AP:R-AP. Although the reason for the existence of different AP isoenzymes is unknown, Schlesinger and Anderson (1968) have reported that the relative proportions of the isoenzymes are dependent on the growth conditions of the bacteria. Thus the aminopeptidase could play a regulatory role in controlling the relative proportions of the AP isoenzymes. Further work on the subcellular localization and enzymic characterization of the aminopeptidase will shed light on the role of this catalyst in the secretion and post-translocational modification of AP in *E. coli*. The identification of the amino peptidase and the approach developed for its inhibition *in vivo* may find an application in the production of secreted forms of recombinant/engineering proteins requiring the preservation of the N-terminal arginine.

We are grateful to the AFRC, the Royal Society and the University of Wales (Research Funds) for supporting this work.

REFERENCES

Barkocy-Gallagher, G. A. and Bassford, P. J. (1992) J. Biol. Chem. **267**, 1231–1238
Black, M. T., Munn, J. G. R. and Allsop, A. E. (1992) Biochem. J. **282**, 539–543
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bridgen, J. and Secher, D. S. (1973) FEBS Lett. **29**, 55–57
Chang, C. N., Kuang, W.-J. and Chen, E. Y. (1986) Nucleic Acids Res. **44**, 121–125
Derman, A. I. and Beckwith, J. (1991) J. Bacteriol. **173**, 7719–7722
Dev, I. J. and Ray, P. H. (1990) J. Bioenerget. Biomembr. **22**, 271–290
Fikes, J. D., Barkocy-Gallagher, G. A., Klapper, D. G. and Bassford, P. J. (1990) J. Biol. Chem. **265**, 3417–3423
Gallagher, J., Kaderbhai, N. and Kaderbhai, M. A. (1992) Appl. Microbiol. Biotechnol. **38**, 77–83
Gallagher, S. R. and Smith, J. A. (1991) In Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kington, R., Moore, R. E., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), vol. 2, pp. 10.0.1–10.2.21, Greene Publishing Associates, New York
Hultquist, D. E. (1978) Methods Enzymol. **52**, 463–473
Karim, A., Harding, V., Evans, A., Kaderbhai, N. N. and Kaderbhai, M. A. (1993) Bio/Technology **11**, 612–618
Li, P., Beckwith, J. and Inouye, H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7685–7689
Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Müller, M. (1992) Experientia **48**, 118–129
Nothwehr, S. F. and Gordon, J. I. (1989) J. Biol. Chem. **264**, 3979–3989
Oroszlan, S. and Luftig, R. B. (1990) Curr. Top. Microbiol. Immunol. **157**, 153–185
Perlman, D. and Halvorson, H. O. (1983) J. Mol. Biol. **167**, 391–409
Sanger, F., Nicklen, S. and Coulson, S. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Schlesinger, M. J. and Anderson, M. (1968) Ann. N.Y. Acad. Sci. **151**, 159–170
Schlesinger, M. J., Block, W. and Kelley, P. M. (1975) in Isoenzymes I: Molecular Structure (Markert, C., ed.), pp. 333–342, Academic Press, New York
von Heijne, G. (1990) J. Membr. Biol. **115**, 195–201
Wickner, W., Driessen, A. J. M. and Hartl, F.-U. (1991) Annu. Rev. Biochem. **60**, 101–124

Received 8 January 1993/2 March 1993; accepted 11 March 1993